Nuclear exclusion of Cdc25 is not required for the DNA damage checkpoint in fission yeast

Antonia Lopez-Girona*, Junko Kanoh*† and Paul Russell

Maintenance of genome integrity requires a checkpoint that restrains mitosis in response to DNA damage [1]. This checkpoint is enforced by Chk1, a protein kinase that targets Cdc25 [2–7]. Phosphorylated Cdc25 associates with 14–3-3 proteins, which appear to occlude a nuclear localization signal (NLS) and thereby inhibit Cdc25 nuclear import [6, 8–14]. Proficient checkpoint arrest is thought to require Cdc25 nuclear exclusion, although definitive evidence for this model is lacking. We have tested this hypothesis in fission yeast. We show that elimination of an NLS in Cdc25 causes Cdc25 nuclear exclusion and a mitotic delay, as predicted by the model. Attachment of an exogenous NLS forces nuclear inclusion of Cdc25 in damaged cells. However, forced nuclear localization of Cdc25 fails to override the damage checkpoint. Thus, nuclear exclusion of Cdc25 is unnecessary for checkpoint enforcement. We propose that direct inhibition of Cdc25 phosphatase activity by Chk1, as demonstrated in vitro with fission yeast and human Chk1 [15, 16], is sufficient for proficient checkpoint regulation of Cdc25 and may be the primary mechanism of checkpoint enforcement in fission yeast.

*These authors contributed equally to this work.

Address: Departments of Molecular and Cell Biology, The Scripps Research Institute, La Jolla, California, 92037, USA.

†Present address: Department of Life Science, Tokyo Institute of Technology, Yokohama 226-8501, Japan.

Correspondence: Paul Russell
E-mail: prussell@scripps.edu

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Results and discussion

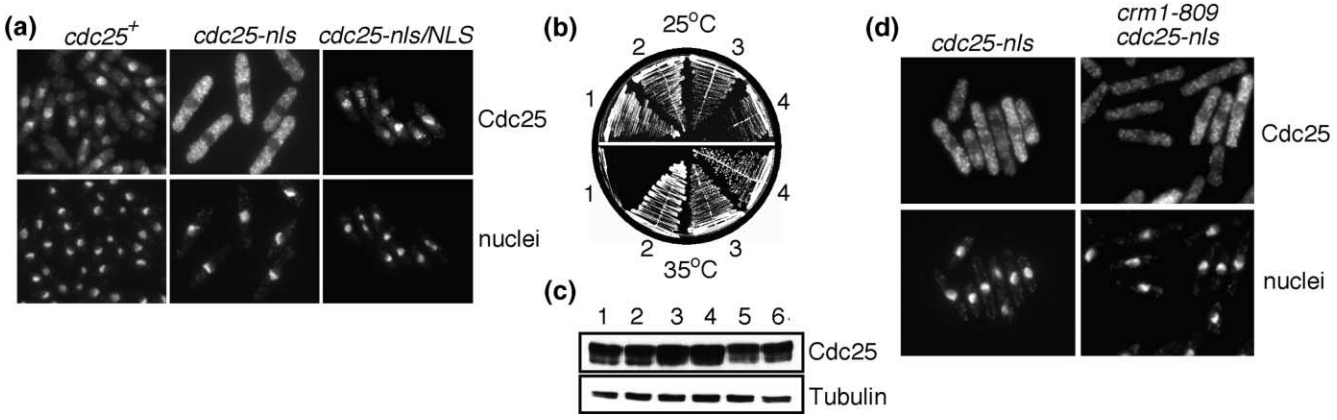
Cdc25 colocalizes in the nucleus with its substrate, Cdc2 bound to cyclin-B, in the fission yeast *Schizosaccharomyces pombe* [9, 17]. The importance of Cdc25 nuclear localization was tested by mutating its presumptive nuclear localization signal (NLS). Three consecutive lysine residues (amino acids 212–214) were deleted to produce the allele *cdc25-nls*. The *cdc25-nls* or *cdc25*$^+$ constructs were integrated in a temperature sensitive *cdc25–22* strain and *cdc25*$^+$ cells. These constructs encoded Cdc25 with a carboxy-terminal "12myc" tag. The integration method was designed to express only the myc-tagged forms of Cdc25 (see Materials and methods). Cdc25 was localized in the nucleus in G_2 cells, whereas Cdc25-nls was excluded from the nucleus (Figure 1a). The *cdc25–22* strain that expressed *cdc25-nls* strain was viable at 35.5°C, demonstrating that Cdc25-nls is functional and Cdc25 nuclear accumulation is not required for mitotic induction (Figure 1b). Immunoblot analysis confirmed that Cdc25 abundance was unaffected by the *cdc25-nls* mutation (Figure 1c).

Nuclear exclusion impaired Cdc25 function in vivo. A *cdc25-nls* strain divided at a cell length of 19.1 ± 1.2 μm, whereas a matched control *cdc25*$^+$ strain divided at 12.1 ± 1.4 μm. The mitotic delay in *cdc25-nls* cells is comparable to that caused by growth of *cdc25–22* cells at the semi-permissive temperature of 29°C, or increasing abundance of the Wee1 mitotic inhibitor ~3-fold [18]. (Wee1 and Mik1 inhibit Cdc2 by phosphorylating tyrosine 15, the residue that is dephosphorylated by Cdc25.)

The mitotic delay observed in *cdc25-nls* cells indicated that Cdc25 nuclear accumulation was important but not essential for Cdc25 function in vivo. However, it was possible that Cdc25-nls was imported weakly into the nucleus at a level that was necessary for mitotic induction but undetectable by immunolocalization. This question was addressed by examining Cdc25-nls localization in a *crm1–809* mutant. Crm1 is an essential exportin protein that is required for the nuclear export of Cdc25 and other proteins [9, 19]. Incubation of *crm1–809* cells at restrictive temperature had no effect on Cdc25-nls localization (Figure 1d), suggesting that Cdc25-nls catalyzes mitosis without entering the nucleus. Cdc25 might dephosphorylate a cytoplasmic form of Cdc2, perhaps relying on nucleocytoplasmic shuttling of Cdc2-cyclin B as demonstrated in vertebrate organisms [20, 21]. It is unknown if Cdc2-cyclin B undergoes nucleocytoplasmic shuttling in fission yeast.

An experiment was devised to confirm that the mitotic delay observed in *cdc25-nls* cells was due to aberrant Cdc25 localization. The NLS from SV40 T-antigen was placed at the carboxy-terminal end of *cdc25-nls* to generate *cdc25-nls/NLS* (see Materials and methods). This construct was integrated as described above. Cdc25-nls/NLS localized in the nucleus, rescued *cdc25–22*, and appeared as abun-

Figure 1

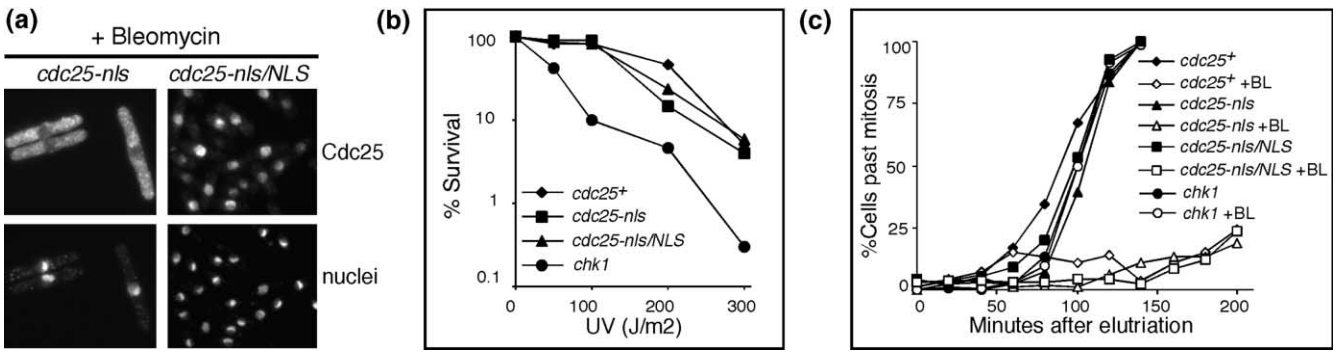


Elimination of the Cdc25 nuclear localization signal (NLS) causes nuclear exclusion of Cdc25. **(a)** Immunolocalization of "12-myc"-tagged Cdc25 proteins expressed from the *cdc25* genomic locus is shown. Cdc25-nls has a mutant NLS and is excluded from the nucleus, whereas Cdc25-nls/NLS has the SV40 T-antigen NLS and appears exclusively nuclear. Nuclei were visualized with DAPI. Cdc25 nuclear staining was decreased in binucleate cells, as previously described [9]. **(b)** A *cdc25–22* strain (1; PR1483) is inviable at 35.5°C, whereas *cdc25–22* strains having integrated plasmids that express Cdc25, Cdc25-nls, or Cdc25-nls/NLS (2–4; AL2660, AL2749, and AL2750, respectively) are viable at 35.5°C. **(c)** An anti-myc immunoblot of 12-myc-tagged cdc25$^+$, cdc25-nls, and cdc25-nls/NLS strains mock-irradiated (lanes 1, 3, and 5, respectively) or exposed to 100 Gy IR (lanes 2, 4, and 6) is shown. **(d)** Inactivation of the exportin Crm1 does not change Cdc25-nls nuclear exclusion. JK2421 (*cdc25-nls:12myc*) and JK2425 (*cdc25-nls:12myc crm1–809*) strains were grown at restrictive temperature (25°C) for 4 hr prior to immunolocalization analysis.

dant as Cdc25 and Cdc25-nls in asynchronous cultures (Figures 1a-c). The *cdc25-nls/NLS* cells divided at 12.8 ± 1.9 μm, a length essentially equivalent to matched control *cdc25*$^+$ cells (12.1 ± 1.4 μm). These findings demonstrated that the mitotic delay in *cdc25-nls* cells was due solely to defective nuclear import of Cdc25.

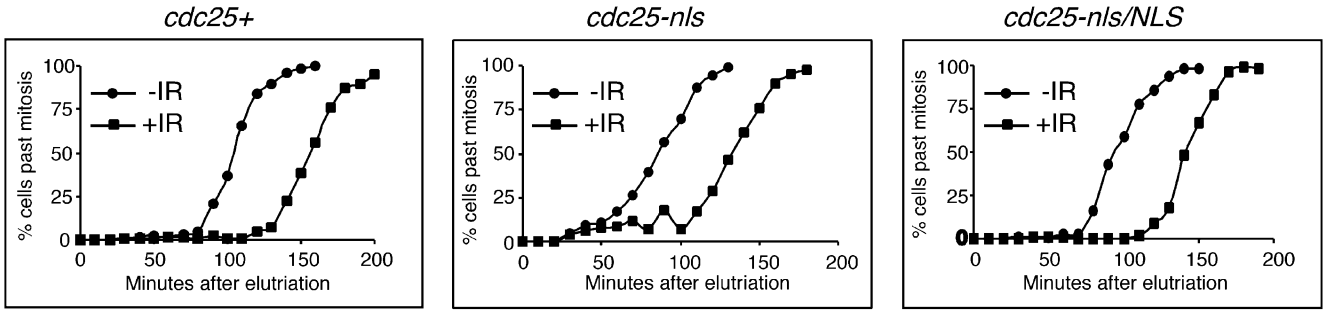
Experiments were performed to determine if Cdc25 nuclear exclusion was necessary for normal survival in response to DNA damage. The DNA damage responses of *cdc25-nls* and *cdc25-nls/NLS* strains were compared. Localization of the two forms of Cdc25 was unaffected by DNA damage caused by the radiomimetic drug bleomycin (Figure 2a). Nuclear exclusion of Cdc25-nls was maintained, whereas Cdc25-nls/NLS remained nuclear. Similar results were obtained with ionizing radiation (IR) (data not shown). Wild-type and mutant forms of Cdc25 were equally abundant in checkpoint-arrested cells (Figure 1c). The *cdc25*$^+$, *cdc25-nls*, and *cdc25-nls/NLS* strains were equally sensitive to ultraviolet radiation and ~10-fold

Figure 2



Forced nuclear inclusion or exclusion of Cdc25 does not abrogate damage checkpoint responses. **(a)** Immunolocalization of Cdc25-nls and Cdc25 nls/NLS are unaffected by DNA damage induced by bleomycin. Bleomycin treatment (45 min) caused cells of both genotypes to undergo cell cycle arrest and become elongated. **(b)** Ultraviolet (UV) survival is unimpaired in cdc25-nls and cdc25 nls/NLS strains. **(c)** Checkpoint delay in *cdc25*$^+$, *cdc25-nls*, and *cdc25-nls/NLS* strains but not Δ*chk1* cells is shown. Cells were synchronized in G_2 phase by elutriation and exposed to 5 mU ml^{-1} bleomycin (+BL) for the duration of the experiment or mock-treated.

Figure 3



Checkpoint regulation of Cdc25 is retained in *cdc25-nls* and *cdc25 nls/NLS* strains. Strains that contained wild-type or mutant *cdc25* in a *wee1–50* Δ*mik1* background were grown at 25°C and synchronized in G_2 phase by centrifugal elutriation. Cells were irradiated (100 Gy) or mock-irradiated and then shifted to 35.5°C. The rate of mitotic induction is determined by Cdc25 activity. Irradiation caused a ~40 min mitotic delay in all strains. Thus, the in vivo function of Cdc25, Cdc25-nls, and Cdc25-nls/NLS was equally inhibited by DNA damage.

more resistant than Δ*chk1* cells (Figure 2b). Similar differences in damage survival were obtained with IR (data not shown). These studies demonstrated that damage survival was unaffected by constitutive nuclear localization of Cdc25.

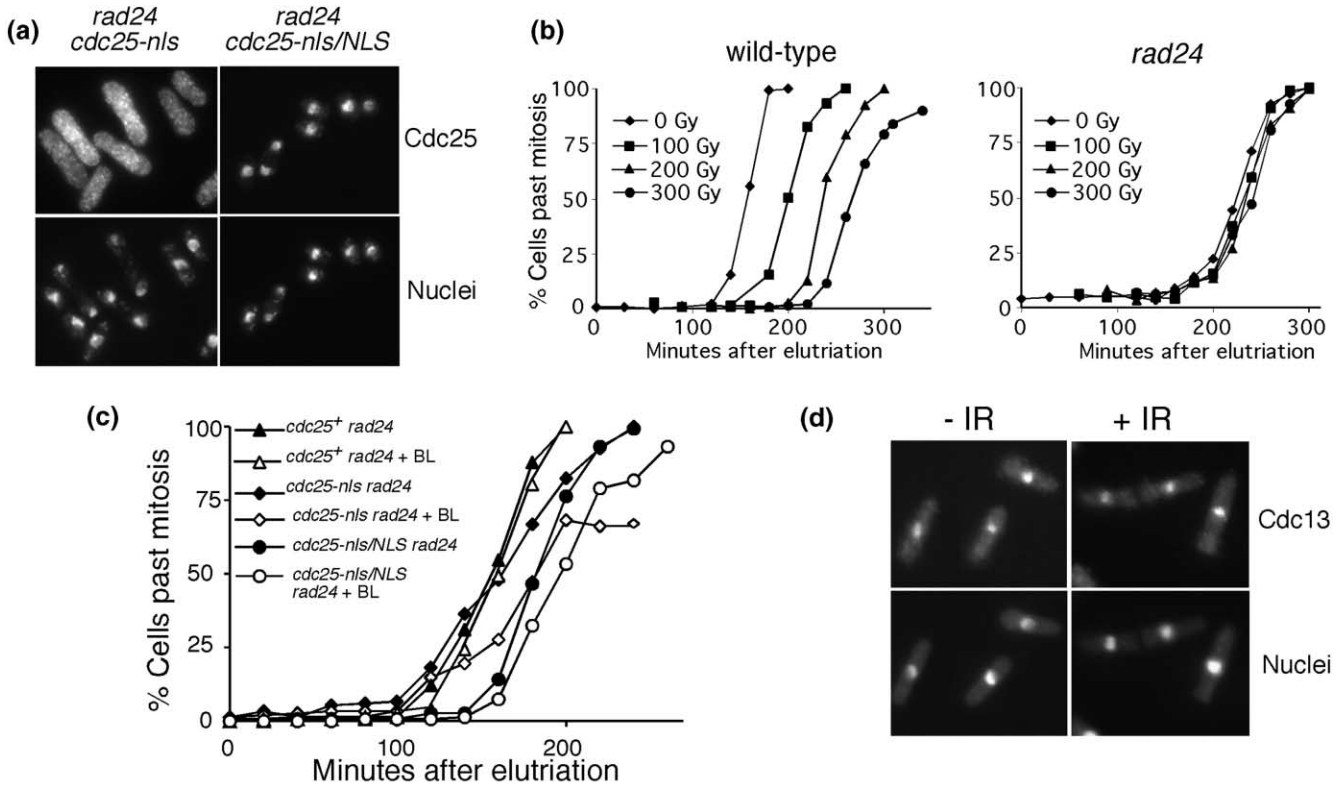
These findings prompted an examination of checkpoint responses in the *cdc25-nls* and *cdc25-nls/NLS* strains. Wild-type (*cdc25*$^+$) and Δ*chk1* strains were included as controls. Synchronized cultures of cells in early G_2 phase were obtained by centrifugal elutriation. Cultures were divided and treated with bleomycin (+BL) or mock treated. Bleomycin elicited a prolonged checkpoint arrest in the *cdc25*$^+$, *cdc25-nls*, and *cdc25-nls/NLS* strains (Figure 2c). As expected, no checkpoint delay was observed in the Δ*chk1* culture. These findings demonstrated that the DNA damage checkpoint did not require nuclear exclusion of Cdc25.

The rate of mitotic induction following inactivation of Wee1 and Mik1 was also measured. The *cdc25*$^+$, *cdc25-nls*, and *cdc25-nls/NLS* alleles were crossed into a *wee1–50* Δ*mik1* strain background. These cells lacked Mik1 and expressed temperature-sensitive Wee1. Incubation of *wee1–50* Δ*mik1* cells at 35.5°C causes Cdc2 tyrosine-15 dephosphorylation and mitotic initiation at a rate determined by Cdc25 activity [4]. Cultures were synchronized in early G_2 by centrifugal elutriation, exposed to 100 Gy IR or mock IR, and the temperature was raised to 35.5°C. IR caused comparable ~40 min mitotic delays in all three strains (Figure 3). Thus, Cdc25, Cdc25-nls, and Cdc25-nls/NLS were equally responsive to checkpoint regulation.

These studies showed that G_2-M DNA damage checkpoint control can occur without nuclear exclusion of Cdc25. What then is the role of 14-3-3 proteins in checkpoint control? 14-3-3 proteins modulate Cdc25 localization and are important for checkpoint enforcement [9–14]. Deletion of *rad24*$^+$, which encodes the 14-3-3 protein required for checkpoint arrest [8], abrogated nuclear exclusion of Cdc25-nls (Figure 4a). This result suggests that 14-3-3 binding might occlude a weak NLS that remains in Cdc25-nls. As expected, nuclear localization of Cdc25-nls/NLS was unaffected by Δ*rad24*. The Δ*rad24* mutation substantially diminishes the checkpoint response to ionizing radiation [8]. We have measured a somewhat more severe effect with our Δ*rad24* strains (Figure 4b). Most importantly, we observed that the Δ*rad24* mutation caused a profound checkpoint defect in *cdc25*$^+$, *cdc25-nls* and *cdc25-nls/NLS* backgrounds (Figure 4b). Thus, Rad24 is important for checkpoint control even when Cdc25-nls/NLS remains localized in the nucleus following DNA damage. Nuclear localization of Cdc25-nls/NLS in *cdc25-nls/NLS rad24*$^+$ cells does not override the damage checkpoint (Figure 2), whereas the checkpoint is severely compromised in *cdc25-nls* Δ*rad24* cells that exhibit comparatively weak nuclear localization of Cdc25-nls. These findings suggest a complex role for Rad24/14-3-3 proteins in checkpoint control. Rad24 interacts with Chk1 and is important for Chk1 phosphorylation [22]; thus, Rad24 may regulate Chk1 activity. Another possibility is that Rad24 protects Cdc25 from phosphatases that counteract phosphorylation catalyzed by Chk1.

These studies establish two important points. First, the cell elongation phenotype of *cdc25-nls* cells demonstrates that nuclear localization of Cdc25 is important for the onset of mitosis, as we predicted [9]. Second, while our experiments show that nuclear exclusion of Cdc25 may contribute to a checkpoint arrest, they also demonstrate that Cdc25 nuclear exclusion is not required for checkpoint inhibition of Cdc25 function in vivo (Figure 3), nor for a robust DNA damage checkpoint arrest (Figure 2c).

Figure 4



14-3-3 proteins are important for checkpoint response irrespective of effects on Cdc25 localization. **(a)** Immunolocalization of Cdc25-nls and Cdc25 nls/NLS in 14-3-3-deficient (Δ*rad24*) strains is shown. **(b)** The checkpoint response to ionizing radiation is abrogated in Δ*rad24* cells exposed to different doses of ionizing radiation. **(c)** Checkpoint delay is abolished in Δ*rad24* strains synchronized in G_2 phase and exposed to bleomycin (+BL). **(d)** Immunolocalization of Cdc13 B-type cyclin in cells exposed to 100 Gy ionizing radiation or mock-treated is shown. Cells were photographed 30 min after irradiation.

It could be argued that Chk1 regulates the localization of another protein and that both proteins must be aberrantly localized to override the damage checkpoint. The most likely candidate is Cdc13, the B-type cyclin that is required for mitosis. However, Cdc13 remains localized in the nucleus in checkpoint-arrested cells (Figure 4d). Moreover, the damage checkpoint is overridden by expression of a mutant form of Cdc25 that cannot be phosphorylated by Chk1 [13]. This result is inconsistent with the proposal that Chk1 causes nuclear exclusion of an unknown protein required for mitotic induction.

If nuclear exclusion of Cdc25 is not required for its checkpoint regulation, then how else can Chk1 regulate Cdc25? Recent in vitro experiments showed that Chk1 potently inhibits activation of Cdc2/cyclin-B by Cdc25 [15, 16]. These studies were performed with both human and fission yeast Chk1. Cdc25 inhibition required Chk1 kinase activity and was reversed by dephosphorylation of Cdc25. In view of the fact that Cdc25-nls/NLS is subject to checkpoint regulation, we propose that direct inhibition of Cdc25 by Chk1 is sufficient for checkpoint control of Cdc25 and is the primary mechanism of enforcing the G_2-M DNA damage checkpoint in fission yeast.

Materials and Methods

Strains

The following strains were used in this study: PR109 (wild type), JK2423 (*cdc25*$^+$*:12myc:ura4*$^+$), JK2421 (*cdc25-nls:12myc:ura4*$^+$), JK2425 (*cdc25-nls:12myc:ura4 crm1–809*), JK2438 (*cdc25-nls/NLS:12myc:ura4*$^+$), PR1483 (*cdc25–22*), AL2660 (*cdc25-nls:12myc:ura4*$^+$), AL2661 (*cdc25*$^+$*:12myc wee1–50 mik1::ura4*$^+$), AL2662 (*cdc25-nls:12myc wee1–50 mik1::ura4*$^+$), AL2663 (*cdc25-nls/NLS:12myc:ura4*$^+$ *wee1–50 mik1::ura4*$^+$), NR1592 (*chk1:ura4*$^+$), AL2664 (*cdc25*$^+$*:12myc:ura4*$^+$ *rad24::ura4*$^+$), AL2665 (*cdc25-nls:12myc:ura4*$^+$ *rad24::ura4*$^+$), and AL2666 (*cdc25-nls/NLS:12myc:ura4*$^+$ *rad24::ura4*$^+$). All strains were *leu1–32*, *ura4-D18*. JK2423, JK2421, and JK2438 were produced by plasmid integration into a *cdc25*$^+$ strain, whereas AL2660, AL2749, and AL2750 were produced by plasmid integration into a *cdc25–22* strain.

Supplementary material

Additional information on strain and plasmid construction and methodological details are available at http://www.current-biology.com/supmat/supmatin.htm.

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